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# Mesenchymal stromal cells in hepatic fibrosis/cirrhosis: from pathogenesis to treatment

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Hepatic fibrosis/cirrhosis is a significant health burden worldwide, resulting in liver failure or hepatocellular carcinoma (HCC) and accounting for many deaths each year. The pathogenesis of hepatic fibrosis/cirrhosis is very complex, which makes treatment challenging. Endogenous mesenchymal stromal cells (MSCs) have been shown to play pivotal roles in the pathogenesis of hepatic fibrosis. Paradoxically, exogenous MSCs have also been used in clinical trials for liver cirrhosis, and their effectiveness has been observed in most completed clinical trials. There are still many issues to be resolved to promote the use of MSCs in the clinic in the future. In this review, we will examine the controversial role of MSCs in the pathogenesis and treatment of hepatic fibrosis/cirrhosis. We also investigated the clinical trials involving MSCs in liver cirrhosis, summarized the parameters that need to be standardized, and discussed how to promote the use of MSCs from a clinical perspective.

**Keywords:** Mesenchymal stromal cells; Hepatic fibrosis/cirrhosis; Pathogenesis; Treatment; Clinical application

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## INTRODUCTION

Liver diseases present a significant threat to human health globally, resulting in ~2 million deaths each year. Hepatic cirrhosis accounts for ~50% of liver disease-associated deaths [1]. Liver cirrhosis is a complication of liver disease, which is a further step toward hepatic fibrosis and involves the loss of liver cells and irreversible liver scarring. Chronic damage due to factors such as viral hepatitis, alcohol, and drugs impairs hepatocytes. This damage induces inflammatory cell infiltration, leading to the excessive accumulation of collagen and extracellular matrix (ECM) and destroying liver structure and function [2, 3]. Activated hepatic stellate cells (HSCs) are the primary source of ECM and contribute to hepatic fibrosis [4]. Increasing evidence has demonstrated that MSCs are involved in the pathological progression of hepatic fibrosis [5, 6] through changes in their functions and microenvironment regulation.

MSCs were first discovered in bone marrow (BM) by Friedenstein and colleagues in 1968 and were described as an adherent, fibroblast-like population in BM in vitro that could differentiate into bone in vivo [7]. Subsequently, numerous studies have revealed the plasticity, high proliferation, multidifferentiation, and immunomodulatory capacity of these cells. MSCs are also present in other tissues, including adipose (AD) tissue, umbilical cord (UC), fetal liver, muscle, and lung [8–12]. MSCs have the ability to differentiate into multiple lineages, such as chondrocytes, osteocytes, adipocytes, myocytes, and astrocytes. MSCs express major histocompatibility complex (MHC) class I but do not express MHC class II, B7-1, B7-2, CD40, or CD40L. MSCs can be expanded more than $10^4$-fold in culture without the loss of their multilineage differentiation potential. Therefore, MSCs could be a potential source of stem cells for cellular and genetic therapy [9, 13].

Exogenous MSCs have been shown to exert antifibrotic effects, and various mechanisms have been examined. MSCs provide an alternative for patients with liver cirrhosis because there is no effective traditional treatment other than liver transplantation, which is expensive and takes a long time to find a suitable organ. MSCs are considered to be the most promising cells for cell therapy due to the following properties: (1) they are easy to obtain from various tissues and easy to expand in vitro without changing their properties [8, 10] (2) they allow for allogeneic transplantation because of their low immunogenicity and lack of ethical issues [14, 15]; (3) they migrate to injury sites; [16] (4) they have multiple differentiation capacity and favorable regeneration and injury repair effects [9, 17]; and (5) they have strong immunoregulatory abilities [18, 19]. There have also been bench studies focusing on new strategies to improve the therapeutic efficacy of MSCs in liver cirrhosis. Although MSCs have been used to treat liver cirrhosis in clinical trials and good outcomes have been observed in most cases, many problems need to be solved to promote the use of MSCs in the clinic in the future. In this review, we aimed to

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examine the different roles of MSCs in the pathogenesis and treatment of hepatic fibrosis/cirrhosis, propose parameters that need to be standardized or optimized and suggest prospects for the use of MSCs to treat hepatic fibrosis/cirrhosis.

## ENDOGENOUS MSCS IN THE PATHOGENESIS OF HEPATIC FIBROSIS

### Contribution of MSCs to the population and regulation of myofibroblasts

*Myofibroblasts: The heart of fibrosis*. Myofibroblasts (MFs) were first described by the seminal works of Gabbiani et al. in 1971, who showed that a population of "modified fibroblasts" was present in granulation tissue during wound healing [20]. The researchers found that these typical fibroblasts (1) possessed fibrillar systems within the cytoplasm; (2) had a deformed nucleus; and (3) were attached to an extracellular layer of basement membrane-like material [20]. Due to their similarities with smooth muscle cells, these unusual fibroblasts were termed muscle-fibroblast intermediate cells and are now referred to as "myofibroblasts" [21]. The presence of MFs in tissue injury led to a series of investigations of their roles in wound repair. Myofibroblast filamentous fibers were found to integrate with myosin and α-smooth muscle actin (α-SMA) proteins through actin [22]. This unique cytoskeleton of MFs allowed them to exert contractile forces and to contract the wound edge, and α-SMA is now regarded as a universal marker of MFs [23, 24]. ECM production is another hallmark of MFs. ECM components, including different types of fibrillar collagens, hyaluronan (HA), fibronectin (FN), and extra domain A fibronectin, are essential for closing wounds [25]. It is important to note that dysregulated wound healing, which frequently occurs in chronic injury, leads to tissue fibrosis. Due to awareness of the essential role of MFs in tissue repair, researchers quickly examined MFs in the context of organ fibrosis. They identified MFs as the primary source of excess ECM deposition in the fibrotic area [26]. Furthermore, multiple pieces of evidence suggest that the status of MFs could balance tissue repair and fibrosis [27]. Therefore, understanding the origin, modulation, and remission of MFs is central to developing treatments for hepatic fibrosis/cirrhosis.

*MSCs are one of the sources of MFs in the liver*. The origins of MFs have long been a central debate in the field. In the late 1970s, MFs were initially thought to be derived from smooth muscle cells [28]. Later, investigators showed that MFs could develop directly from normal fibroblasts, MSCs, epithelial cells, pericytes, and circulating fibrocytes [29]. These contradictory findings complicated the field, and a lack of consensus on the sources of MFs was a significant obstacle to understanding organ fibrosis. The groundbreaking technological advances in lineage tracing and single-cell analysis have enabled the detailed characterization of MFs under homeostatic and disease conditions. The latest conceptual advance on MFs is that they are a heterogeneous population of cells whose origins and properties differ in various organs. The following section focuses on the relationship between MSCs and MFs during hepatic fibrosis.

The notion that MSCs were a source of MFs first came from in vitro studies [5]. Transforming growth factor β (TGF-β), which is a major profibrotic cytokine, was shown to induce α-SMA expression in MSCs. These α-SMA$^+$ MSCs produce ECM, validating the transition of MSCs to MFs. Notably, the inflammatory properties of MSCs suggest that these in vitro observations may also occur under pathological conditions. Taking advantage of a dual fluorescent labeling system, Michael Quante et al. found that BM-derived MSCs were recruited to the site of chronic inflammation and gave rise to α-SMA$^+$ MFs during *Helicobacter felis*-induced gastric cancer [6]. The spatiotemporal coexistence of BM-MSCs and TGF-β during liver injury repair was likely to produce BM-MSC-derived hepatic MFs. In addition to BM niches, MSCs are found in almost all tissues. These tissue-resident MSCs are located in perivascular niches and are capable of trilineage differentiation in vivo [30]. Recent studies using lineage tracing in mice showed that Gli1 is a marker for resident MSCs in the liver, kidney, lung, heart, and muscle [30]. Gli1$^+$ MSCs constitute 0.02% of total live cells in the liver. These liver-resident MSCs also express CD29, CD44, CD105, Sca1, and CD34. During carbon tetrachloride ($CCl_4$)-induced hepatic fibrosis, hepatic Gli1$^+$ MSCs dramatically expand and induce the expression of α-SMA in a TGF-β-dependent manner [30]. Notably, Gli1$^+$ MSCs contribute to ~39% of interstitial α-SMA$^+$ cells in hepatic fibrosis in Gli1-CreERt2-tdTomato tracing mice. Ablation of Gli1$^+$ MSCs was reported to alleviate the severity of tissue fibrosis.

*MSCs regulate myofibroblast generation from hepatic stellate cells*. While the contribution of MSCs to the hepatic myofibroblast pool was recently validated, it is generally believed that HSCs are the primary sources of MFs during hepatic fibrosis. HSCs are pericyte-like cells with abundant intracellular vitamin A and lipids [31]. Fate mapping studies using acyltransferase-Cre mice revealed that more than 80% of hepatic MFs were derived from HSCs [32]. When we combine the data on Gli1$^+$ MSCs and HSCs, there is a potential paradox: the sum of MSC-derived MFs and HSCs exceeds 100%. A possible explanation for this contradiction is that MSCs and HSCs have overlapping subpopulations. HSCs expanded in vitro express MSC-like surface markers and show adipogenic and osteogenic differentiation potential [33]. Future studies are required to delineate the similarities between hepatic MSCs and HSCs.

HSCs are nonproliferative in the steady state, and MF differentiation in these cells is initiated by inflammatory cytokines released by injured hepatocytes or immune cells. MSCs have been reported to regulate the activation of HSCs. MSCs have been reported to express soluble and membrane-bound TGF-β, which may provide signals for the myofibroblast differentiation of HSCs [34]. However, MSCs were shown to directly suppress the proliferation of HSCs by the surface expression of Notch1, which downregulates the PI3K/AKT pathway in HSCs in an in vitro coculture system [35]. It has also been reported that direct contact with MSCs induces the expression of proapoptotic proteins, such as Bax and cleaved caspase-3, in HSCs [36]. In addition to cell–cell contact, MSCs may affect HSCs through paracrine effects. Hepatocyte growth factor (HGF) and nerve growth factor (NGF), which are secreted by MSCs, were shown to impair the activation of HSCs by inhibiting nuclear factor kappa B (NF-κB) signaling [37]. The rationale for these in vitro findings is that MSCs and HSCs are both located in perivascular niches, allowing for potential cell–cell communication or paracrine effects. However, the reciprocal interactions between MSCs and HSCs during the pathogenesis of hepatic fibrosis remain to be determined.

### Immunomodulation of MSCs in hepatic fibrosis

Investigations of stem cells have primarily focused on elucidating the mechanisms underlying their self-renewal and differentiation properties. However, most studies of MSCs have been related to their roles in immune regulation [38]. The first paper linking MSCs with immunoregulation comes from the observation that the presence of MSCs effectively suppressed mixed lymphocyte reactions [39]. The immunosuppressive capabilities of MSCs were then firmly validated by the successful clinical application of MSCs to treat a severe steroid-resistant GvHD patient in 2004 [40]. However, when MSC-mediated immunosuppression was intensively studied, controversial results also emerged. The first FDA-approved MSC-based clinical trial to treat GvHD failed to achieve significant clinical improvements [41]. Later, it was found that the immunosuppressive effects of MSCs were not intrinsic but were triggered by inflammatory cytokines [42, 43]. Depending on the

context of the cytokine milieu, MSCs can exert proinflammatory or anti-inflammatory effects by modulating adaptive and innate immune cells [38]. Based on the concept that MSCs are plastic in the context of immunoregulation, we will discuss how the interaction of MSCs with different types of immune cells affects the progression of hepatic fibrosis.

*Initiation of early inflammation in liver injury*. Hepatic fibrosis is a dysregulated wound-healing response to liver injury [44]. If not appropriately controlled, the acute phase of liver injury is the starting point of hepatic fibrosis. Damage-associated molecular patterns (DAMPs) released from apoptotic or necrotic hepatocytes initiate the inflammatory response by recruiting innate immune cells to the site of tissue injury by secreting chemokines, including C-C motif chemokine ligand 2 (CCL2), C-C motif chemokine ligand 5 (CCL5), C-X-C motif ligand 1, and C-X-C motif ligand 15 [45]. HSCs and liver-resident macrophages are believed to be the sources of chemokines induced by liver injury [45]. MSCs are also immune sentinels that produce chemokines in response to danger signals or cytokines in vivo [46, 47]. It has been reported that BM-MSCs can sense low levels of circulating microbial molecules and mediate monocyte mobilization by producing CCL2 [46]. Notably, most types of liver insult are due to viral infection, alcohol, and nonalcoholic steatohepatitis (NASH), which do not yield high levels of danger signals [44]. In this context, the possibility that liver-resident MSCs initiate inflammation in response to liver injury should be considered since the signaling threshold for chemokine induction is low in MSCs [48].

Neutrophils are the first leukocytes to respond to chemokines released from the injured liver, and the infiltration of neutrophils is commonly observed in patients with steatohepatitis [49, 50]. Generally, neutrophils have a short half-life [51]. However, the viability of neutrophils is increased in the presence of MSCs [52]. MSCs constitutively express interleukin 6 (IL-6) and granulocyte-macrophage colony-stimulating factors to inhibit neutrophil apoptosis without affecting the phagocytic function of neutrophils [52, 53]. The postponed death of neutrophils provides a window to eliminate damaged hepatocytes, which is energy efficient from an evolutionary perspective.

*Resolution of liver inflammation*. Acute immune responses corresponding to the accumulation of neutrophils and T cells lead to the abundant production of inflammatory cytokines in the liver [54]. Activated tissue-resident natural killer T cells and newly arrived neutrophils express high levels of interferon-gamma (IFN-γ), IL-1, and tumor necrosis factor α (TNF-α) [55–57]. It has been shown that MSCs are highly immunosuppressive in response to proinflammatory cues [58]. Treatment of MSCs with a combination of IFN-γ and TNF-α or IL-1β induced massive production of immunosuppressive molecules, including nitric oxide (NO) in rodent MSCs and indoleamine 2,3-dioxygenase (IDO) in humans [43, 59, 60], as well as prostaglandin E2 (PGE2) [61] and TGF-β [62], leading to the subsequent inhibition of T-cell proliferation and the induction of anti-inflammatory innate immune cells. The immunosuppressive molecules and the apparent chemokine gradient surrounding activated MSCs synergistically form MSC-centered immunosuppressive niches in liver tissue. Indeed, intravital imaging showed that MSCs colocalized with macrophages in hepatic fibrosis [63]. The inflammation-induced immunosuppressive functions of MSCs were thought to reshape the immune microenvironment of the liver during injury. However, due to a lack of specific markers for MSCs and their activation status, the MSC-centered immunosuppressive population during hepatic fibrosis has not been elucidated in vivo. Recent advances in mass spectrometry and imaging are promising for investigating the localization-dependent immunosuppressive capabilities of MSCs in hepatic fibrosis [64].

To re-establish immune homeostasis in inflammatory tissues, MSCs have been shown to enhance apoptosis in T cells, suppress the differentiation of T helper 17 (Th17) cells, and induce the generation and accumulation of regulatory T (Treg) cells through high expression of inducible nitric oxide synthase (iNOS), IDO, tumor necrosis factor-stimulated gene-6 (TSG6) and matrix metalloproteinases (MMPs) [43, 48, 65, 66]. These observations were consistent with the significant increase in Treg cells and a decrease in Th17 cell infiltration in fibrotic liver tissues after the transfusion of MSCs [67, 68]. It was reported that the presence of IL-17 could enhance the stability of iNOS and programmed death ligand 1 (PD-L1) mRNA by modulating ARE/poly (U)-binding/degradation factor 1 (AUF1), thus promoting the immunosuppressive abilities of MSCs [69]. Therefore, MSCs activated by acute inflammatory cues can control excessive immune responses and sustain tissue homeostasis.

In addition to T cells, macrophages play essential roles in immune homeostasis in the liver. Recent studies have shown that MSCs can impart anti-inflammatory properties to macrophages during the monocyte-to-macrophage transition [70]. Specifically, these effects depend on insulin-like growth factor 2 (IGF2) production by MSCs. IGF2 preprograms maturing macrophages toward metabolic commitment to oxidative phosphorylation (OXPHOS) [70]. Even in the presence of proinflammatory cues, IGF2-preprogrammed macrophages could exert vital anti-inflammatory effects by upregulating PD-L1 expression [70]. Therefore, the anti-inflammatory macrophages trained by MSC-derived IGF2 could be another layer in the mechanism that quenches inflammation during liver injury. Notably, IGF2 has a dose-dependent effect on training macrophages. At low concentrations, IGF2 prefers to bind the IGF2 receptor on monocytes to confer macrophages with anti-inflammatory properties [71]. However, when the amount of IGF2 was sufficient to ligate the IGF1 receptor on monocytes, the resultant macrophages were proinflammatory. The balance between IGF2 receptor and IGF1 receptor signaling is critical in determining the functions of macrophages.

*Maintenance of chronic inflammation in hepatic fibrosis*. During the chronic inflammation phase, low levels of inflammatory stimuli induce MSCs to continuously secrete chemokines and recruit immune cells to the liver microenvironment [43, 48, 72]. It was reported that iNOS and IDO act as switches in MSC-mediated immunomodulation, and the stimulation intensity triggers a switch between pro- and anti-inflammatory functions [48, 59]. When iNOS in murine MSCs or IDO in human MSCs was genetically ablated, the immunosuppressive effects of IFN-γ- and TNF-α-treated MSCs were diminished, and a significant immunostimulatory effect was observed [48, 59]. As we have previously discussed, potent inflammation in the acute phase initiates the immunosuppressive functions of MSCs. While the cytokines in liver tissue are inadequate to induce the expression of iNOS or IDO, these MSCs can be activated under suboptimal conditions and secrete chemokines, such as C-X-C motif ligand 9 (CXCL9) and CCL5, which enables them with immunostimulatory capabilities [43, 48]. The dual functions of MSCs in immunomodulation might partially account for the chronic inflammation during the progression of hepatic fibrosis.

TGF-β is a major pathogenic cytokine in hepatic fibrosis that is responsible for MF induction. However, TGF-β also plays an essential role in immunosuppression, inhibiting the activation of immune cells and inducing the generation of Treg cells [73]. However, why TGF-β signaling fails to resolve chronic inflammation in the liver remains unclear. Interestingly, investigators found that TGF-β may promote immune responses through interactions with MSCs [74]. TGF-β could induce the immunosuppressive effects of MSCs by inhibiting inflammatory cytokine-induced iNOS expression in a SMAD3-dependent manner, thus sustaining inflammation.

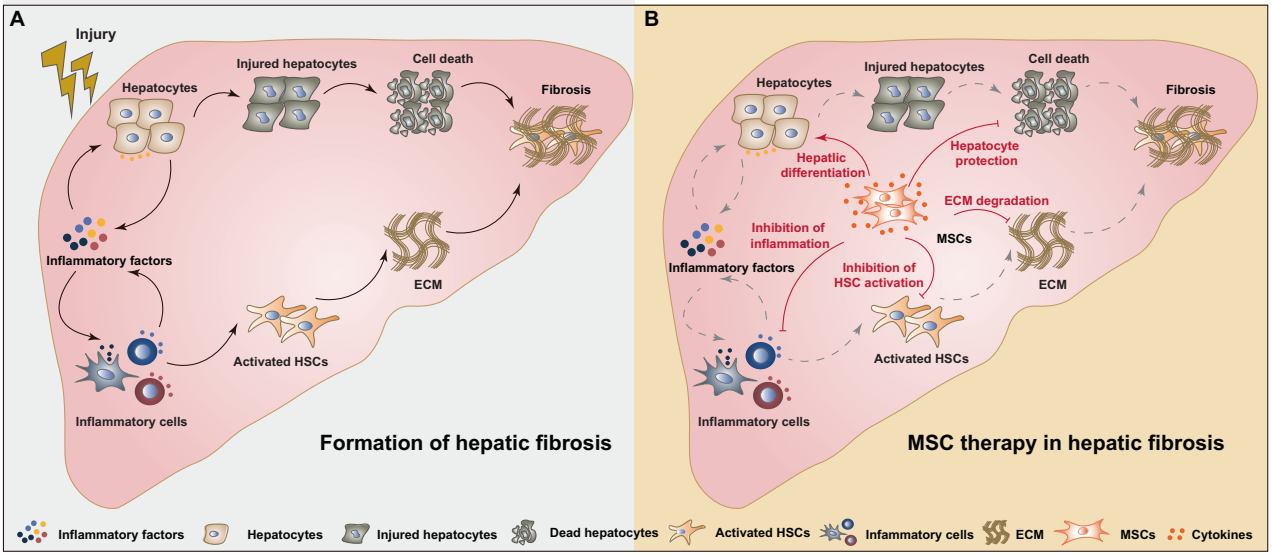


**Fig. 1** The role of MSCs in hepatic fibrosis formation and therapy. **A** In the process of chronic inflammatory injury of liver, the synergistic effect from inflammation and the death of hepatocytes promotes the formation of hepatic fibrosis. **B** The mechanisms of MSC therapy in hepatic fibrosis, including hepatic differentiation, hepatocyte protection, inhibition of HSC activation, ECM degradation and inflammation inhibition

## THERAPEUTIC MECHANISMS OF EXOGENOUS MSCS IN HEPATIC FIBROSIS

As we summarized previously, liver injury-related factors, including viruses and alcohol, can lead to hepatocyte damage and chronic inflammation in the liver. During chronic inflammatory injury of the liver, the synergistic effects of inflammation and hepatocyte death promote the development of hepatic fibrosis. The role and mechanism of MSCs in the treatment of hepatic fibrosis are thoroughly discussed in this section (Fig. 1).

### MSCs in the context of hepatocyte injury

*Differentiation into hepatocytes*. Continuous hepatocyte death is an essential pathological phenomenon in hepatic fibrosis. Due to the multidifferentiation potential of MSCs, targeting the hepatic differentiation potential of MSCs is expected to become an alternative strategy for treating hepatic fibrosis. Several studies have shown the hepatic differentiation of MSCs in experimental animal models and humans [75–78]. For example, after direct xenografting into allyl alcohol (AA)-treated rat livers, human bone marrow-derived MSCs could differentiate into hepatocyte-like cells [79]. After being infused into mice with $CCl_4$-induced liver injury, MSCs or MSC-derived hepatocytes were found in the liver [80]. The liver tropism of MSCs in response to hepatocyte injury is regulated by the coordinated effects of growth factors and damage signals [81]. HGF is the initial driving force that recruits MSCs to the liver. The migration of MSCs is terminated by adenosine, a metabolite that indicates tissue damage, thus retaining MSCs in the area of hepatocyte injury [81]. The transplantation of MSC-derived hepatocyte-like cells could effectively attenuate hepatic fibrosis and protect liver function [82].

It has been reported that cocktail treatment of cytokines, including HGF, epidermal growth factor (EGF), fibroblast growth factor (FGF), leukemia inhibitory factor, dexamethasone (DEX), and nicotinamide could effectively induce the differentiation of MSCs toward hepatocyte-like cells [83–86]. Coculture with liver cells or pellet culture can also lead to the differentiation of MSCs into hepatocytes [87, 88]. In the absence or presence of growth factors, a dynamic cultured scaffold stimulated MSCs to express endodermal and hepatocyte-specific genes and proteins associated with improved functions, and the cells exhibited the ultrastructural characteristics of mature hepatocytes [77]. In addition, Maryam et al. demonstrated that Wharton's jelly-derived MSCs could differentiate into hepatocyte-like cells by permeabilization in the presence of HepG2 cell extracts [89].

However, compared with their differentiation into hepatocytes, the differentiation efficiency of MSCs into MFs is higher, which limits their application value in clinical therapy. However, the transdifferentiation of MSCs into hepatocytes has rarely been observed in animal models [90]. Therefore, it is necessary to examine a more efficient method to induce the differentiation of MSCs into hepatocytes. On the one hand, the biomimetic microenvironment constructed by human E-cadherin fusion protein (hE-cad-Fc)-coated poly (lactic-co-glycolic acid) (PLGA) microparticles (hE-cad-PLGAs) in engineered multicellular aggregates was able to promote endoderm differentiation and the subsequent hepatic differentiation of human MSCs [91]. On the other hand, Wang et al. found that the viability and hepatogenic differentiation of BM-MSCs cultured on plates coated with Matrigel and ECM were significantly enhanced compared with those of cells cultured on noncoated plates [92]. Posttranscriptional regulation also plays an important role in regulating the differentiation of MSCs into hepatocytes. Zhou et al. screened the best miRNA candidates for hepatocyte differentiation and found that five miRNAs, including miR-122, miR-148a, miR-424, miR-542-5p, and miR-1246, were essential for hepatocyte differentiation because omitting anyone from the five miRNAs prevented hepatic transdifferentiation [93].

*Protection of injured hepatocytes*. Increasing evidence has shown that the production of trophic factors, including cytokines, chemokines, and growth factors, is essential for MSCs to contribute to damage repair. Most injured hepatocytes undergo cell death. However, the distinct forms of cell death and cell death response pathways used by damaged hepatocytes have long-term consequences on liver repair [94]. Another pathway by which MSCs participate in liver damage repair involves the production of paracrine trophic factors by MSCs through the paracrine pathway [95, 96]. It has been reported that MSC-derived conditioned medium (CM) effectively inhibits hepatocyte death and promotes liver regeneration [97]. The administration of MSC-CM to rats with acute liver injury dramatically prevents apoptotic hepatocellular death [97]. HGF, αFGF, EGF, and TGF-α contribute to hepatocyte proliferation, and vascular endothelial growth factor (VEGF) is the most vital pro-vascular

growth factor that plays a crucial role in angiogenesis during liver regeneration [98–101]. Gokhan Adas et al. showed that after the injection of MSCs and VEGF-transfected MSCs into the portal vein following liver resection, the cells engrafted in the liver, increased bile duct and hepatocyte proliferation and secreted many growth factors, including HGF, TGF-β, VEGF, platelet-derived growth factor, EGF, and FGF, via paracrine effects [102]. In addition, hepatic progenitor cells (HPCs), which are located in the canals of Hering, are also involved in liver damage repair by differentiating toward hepatocytes or biliary lineage cells. EGF and HGF have been reported to play important roles in HPC differentiation [103]. In addition, MSC-derived exosomes were shown to suppress ferroptosis in $CCl_4$-induced acute liver injury. Mechanistically, MSC-derived exosomes stabilized the protein level of SLC7A11 in primary hepatocytes to downregulate prostaglandin-endoperoxide synthase 2 and lipoxygenases [104]. Interestingly, MSCs were also shown to protect hepatocytes by promoting hepatocyte autophagy through paracrine effects. Let-7a-5p, which is enriched in MSC-derived exosomes, can activate autophagy by targeting mitogen-activated protein kinase kinase kinase kinase 3 (MAP4K3) in hepatocytes [105]. MSCs can also exert hepatoprotective effects against liver damage by upregulating PTEN-induced kinase 1-dependent mitophagy [106]. Therefore, MSCs could shift hepatocyte death toward an ordered mode to limit tissue damage and inflammation, thus reducing the incidence of hepatic fibrosis.

### Inhibition of HSC activation

Studies on fibrosis have always used the strategy of inhibiting the activation of HSCs to achieve an antifibrotic effect. Therefore, the impact of MSCs on HSC activation is particularly important for the effective treatment of hepatic fibrosis. It has been reported that in a $CCl_4$-induced rat hepatic fibrosis model, MSC transplantation effectively ameliorated hepatic fibrosis by inhibiting HSC proliferation and promoting HSC apoptosis [107]. Several studies confirmed the suppressive effect of MSCs on HSCs. TGF-β3 and HGF derived from MSCs lead to arrest in the G0/G1 phase in HSCs by decreasing extracellular signal-regulated kinase 1/2 phosphorylation and thereby ameliorating hepatic fibrosis [108]. MSCs can also decrease HSC proliferation by activating the Notch signaling pathway and downregulating the PI3K/AKT signaling pathway [35]. It has also been reported that hUC-MSCs inhibit the proliferation of HSCs, possibly by inhibiting TGF-β1 and Smad3 expression and increasing Smad7 protein expression [109]. In addition, MSCs could induce apoptosis in HSCs. Transfer of circDIDO1 mediated by MSC-derived exosomes suppresses proliferation, reduces profibrotic markers, and induces apoptosis and cell cycle arrest in HSCs through the miR-141-3p/PTEN/AKT pathway [110]. Lin et al. demonstrated that NGF produced by MSCs increased HSC apoptosis via the NF-κB- and BCL-xl-associated signaling pathways [111]. In addition, HSC activation is triggered by the inflammatory microenvironment. MSCs are well known for their immunosuppressive properties, and MSCs can also alleviate the expression of inflammatory factors that induce HSC activation and fibrosis [112].

On the other hand, MSCs play key roles in ECM degradation. MFs can internalize MSC-derived extracellular vesicles (EVs) to reduce the mRNA levels of type I collagen, thus decreasing ECM production by MFs [113]. Analysis of the components of EVs suggested that these effects were likely mediated by miR-21 and miR-29c, which target the signaling hubs for ECM production [113]. In addition, MSCs also produce ECM-remodeling enzymes, including MMP2, MMP9, and their inhibitors tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1 and TIMP-2), to reduce ECM deposition in fibrotic sites [114]. However, there are controversial results showing that the activities of MMP-2, MMP-9, and MMP-13 were reduced in cardiac fibrosis after MSC treatment [115]. This effect could be due to the context-specific regulation of MMPs by MSCs. Janina Burk et al. cultured MSCs on different ECM substrates and found that fibrotic-like ECM reduced MMP production by MSCs [116].

### Inflammatory microenvironment regulation by MSCs

Accumulating evidence has shown that the immunomodulatory potential of MSCs plays an important role in alleviating hepatic fibrosis. MSCs demonstrated immunosuppressive potential and effects on innate and adaptive immune responses through cell contact or the secretion of immunoregulatory factors, including heme oxygenase-1 (HO-1), NO, PGE2, IDO, IL-6, and HLA-G5 [117–122]. Soluble factors are constitutively produced by MSCs or released after MSC crosstalk with target cells. On the one hand, these immunoregulatory factors can reduce liver injury caused by inflammation. On the other hand, they also provide a suitable microenvironment for liver damage repair. MSCs can regulate several kinds of immune cells, such as T and B lymphocytes, natural killer (NK) cells, macrophages, Treg cells, neutrophils, and myeloid-derived suppressor cells (MDSCs). Kazuya Sato et al. demonstrated that NO produced by MSCs is one of the major mediators of T-cell suppression by MSCs [120]. iNOS deficiency in MSCs abolishes the therapeutic effects of MSCs in advanced hepatic fibrosis models [123]. In addition, it has been reported that MSC-derived PGE2 and IDO mediate the immunosuppressive potential of MSCs. On the one hand, PGE2 effectively mediates the inhibition of NK cells by MSCs [124]. On the other hand, PGE2 and IDO enhance Treg and Th17 cell differentiation and inhibit Th1 cell differentiation [125, 126]. It has also been reported that MSCs induce the differentiation of Th17 cells toward Treg cells by producing HGF, which generates an immunosuppressive environment [68].

Macrophages are critical inflammation-related cells in the hepatic inflammatory microenvironment that play important roles in regulating the hepatic inflammatory microenvironment. Anoop Babu Vasandan et al. found that MSC-derived PGE2 manipulates metabolic programs in differentially polarized macrophages to re-educate these cells [127]. MSCs can induce the differentiation of macrophages to immunosuppressive phenotypes through direct interactions [128]. MSC-derived IL-6 plays a crucial role in repressing M1 polarization during inflammation and promoting M2b polarization under anti-inflammatory conditions [121].

Augello et al. found that direct contact between MSCs and target cells in a cognate fashion inhibited cell proliferation via the engagement of the inhibitory molecule programmed death 1 with its ligands PD-L1 and PD-L2, leading the target cells to modulate the expression of different cytokine receptors and transduction molecules associated with cytokine signaling [129]. MSCs suppress the activation of $CD4^+$ T cells, downregulate IL-2 secretion, and induce irreversible hyporesponsiveness and cell death via the expression and secretion of PD-L1 and PD-L2 [130].

## BENCH RESEARCH ON IMPROVING THE THERAPEUTIC EFFECT OF MSCS ON HEPATIC FIBROSIS

### Pretreatment of MSCs

The therapeutic effects of MSCs can be improved by changing the cell culture conditions to further modulate the homing capacity, survival, hepatogenic differentiation, and paracrine effects of MSCs. Different attempts have been made to improve the efficacy of MSCs by modifying the culture conditions, including changing the culture media with additives, such as growth factors, chemical agents, lipids, vitamins, and inflammatory factors, and other conditions, such as hypoxia. Pretreatment of MSCs with HGF and FGF4 before transplantation improved MSC homing, enhanced the transdifferentiation of MSCs into hepatocyte-like cells in the recipient mouse liver and increased the therapeutic effects on $CCl_4$-induced fibrosis [131]. Melatonin-pretreated MSCs showed a

high homing capacity to injured liver sites. These cells significantly improved the percentage of glycogen storage and decreased collagen and lipid accumulation in fibrotic liver tissue [132]. Fathy et al. showed that eugenol significantly improved the self-renewal, migration, and proliferation characteristics of AD-MSCs in vitro and greatly enhanced the therapeutic effects against hepatic fibrosis induced by $CCl_4$ in rats [133]. Another recent study reported that vitamin E pretreatment improved the homing of transplanted Wharton's jelly-derived allogenic MSCs, prevented oxidative stress, and alleviated hepatic fibrosis [134]. L-theanine pretreatment promoted anti-inflammatory and antiapoptotic and HGF secretion by AD-MSCs, which decreased the fibrosis level in the N-nitrosodiethylamine-induced liver injury model [135]. TLR4- and IFN-γ-activated MSCs reduced fibrosis-related actin alpha 2 (ACTA2), collagen type I alpha 1 chain (COL1A1), TGF-β, and TNF levels in the liver. TLR4- and IFN-γ-activated MSCs could enhance the Th1-polarized response, which alleviated liver granulomatous and fibrosis in mice infected with *Schistosoma japonicum* [136].

**Gene modification of MSCs**

Gene modification of MSCs through viral or nonviral vectors has been demonstrated to improve stemness, differentiation, immunoregulation, homing capacity, and other repair-related abilities in vitro and in vivo. A series of genes have been overexpressed in MSCs; among them, HGF was broadly used to modify MSCs and improve the therapeutic effects on hepatic fibrosis, since HGF has been shown to play a critical role in liver regeneration [137–140]. Other genes, such as IGF1, hepatic nuclear factor (HNF) 4α, FGF4, FGF21, IL-10, SERPINA1, PTP4A1, SMAD7, and ECM1, have been used to modify MSCs and showed improved therapeutic effects in the treatment of hepatic fibrosis (Table 1). HNF4α-overexpressing MSCs inhibited inflammation by enhancing iNOS expression through the NF-κB signaling pathway in a $CCl_4$-induced mouse liver injury model, ultimately reducing inflammation in the liver and alleviating hepatic fibrosis [141]. Forkhead box A2 (FOXA2)-overexpressing MSCs promoted the recovery of fibrotic liver tissue by enhancing hepatogenic differentiation in MSCs and upregulating the expression of liver-specific genes, including FOXA2, alpha fetoprotein, keratin 18 (CK-18), HNF1α, and HGF [142]. ECM1-overexpressing hair follicle-derived MSCs could had significantly improved the homing and differentiation into hepatocytes, which increased liver function and decreased pathological liver injury in liver cirrhosis. In addition, HSC activation was significantly inhibited in the MSC treatment groups in vivo and in vitro [143]. In addition to the overexpression of target genes, modifications of microRNAs could also improve the effects of MSCs. MiR-122 modification enhanced the therapeutic efficacy of AD-MSCs in the treatment of hepatic fibrosis by suppressing the activation of HSCs and alleviating collagen deposition [144].

Furthermore, genetically modified MSCs can enhance the immunomodulation of MSCs, thus increasing the therapeutic effect of MSCs. IL-10 gene-edited amniotic MSCs inhibited the activation of HSCs and TNF-α expression in T cells/macrophages derived from thioacetamide (TAA)-induced fibrotic livers [145]. Another study indicated that IL-35-overexpressing MSCs had increased immunosuppressive capabilities, which caused $CD4^+$ T cells to produce IL-10 [146]. Therefore, modifying specific genes in MSCs is a potential new strategy for improving their ability to treat hepatic fibrosis.

**MSC spheroids**

There are significant differences in cell phenotypes and biological activities in three-dimensional (3D) and two-dimensional (2D) cell cultures. MSC spheroid culture has been reported to increase the therapeutic potential of MSCs [147]. Studies have demonstrated that MSC spheroids have robustly enhanced therapeutic potential by improvements in MSC stemness, multipotent differentiation, anti-inflammatory effects, angiogenesis, and tissue regeneration through the secretion of high levels of cytokines. AD-MSC spheroid formation promotes stemness, including self-renewal and multidifferentiation capacities, and spheroid-derived AD-MSCs show improved potential to attenuate liver failure [148]. In an experimental model, UC-MSC-3D treatment exhibited enhanced hepatogenic differentiation and improved the functional recovery of the liver with marked decreases in ALT and AST and a mild increase in albumin in a murine model of $CCl_4$-induced liver cirrhosis [149]. Another study demonstrated that AD-MSC spheroids had enhanced stemness and anti-inflammatory and immunomodulatory functions, as revealed by the increased expression of the stem cell markers OCT4, SOX2, and NANOG, the anti-inflammatory factors IL-10, TSG6, and IDO, and the immunomodulatory molecules HGF, VEGF, and C-X-C motif chemokine receptor 4 (CXCR4) [150]. It was also suggested that 3D-cultured MSC spheroids significantly promoted wound healing through well-organized collagen fibrils and high expression of the angiogenesis biomarker CD31 [151]. In addition, stem cells from human exfoliated deciduous teeth (SHED) are an ideal source of MSCs. SHED-converted hepatocyte-like cell-based spheroids improved liver dysfunction in association with antifibrotic effects in $CCl_4$-treated mice [152]. Taken together, these findings suggest that MSC spheroids may replace MSCs as a novel therapeutic strategy with excellent application prospects to treat hepatic fibrosis.

**MSC sheets**

Okano's team developed a cell sheet engineering technology using thermoresponsive culture dishes coated with poly(N-isopropyl acrylamide) [153]. IC-2, which is a derivative of the Wnt/β-catenin signaling inhibitor ICG-001, efficiently induced hepatic differentiation in human MSCs. A new therapy combining IC-2 treatment and MSC sheet technology was developed to establish a novel therapeutic strategy for liver diseases. Research has reported that the transplantation of IC-2-engineered BM-MSC sheets exerted potent regenerative effects and effectively improved acute liver injury in mice [154]. IC-2 also promoted MMP-14 secretion by BM-MSCs from elderly patients. Secreted MMP-14 was a valuable predictive factor in reducing hepatic fibrosis. In addition, MMP-13 activity and thioredoxin levels in IC-2 sheets were inversely correlated with hepatic hydroxyproline levels [155]. Similar effects were also evident in a study by Itaba et al., which indicated that orthotopic transplantation of IC-2-engineered MSC sheets markedly reduced hepatic fibrosis induced by chronic $CCl_4$ administration. Mechanistically, IC-2-engineered MSC sheets produced high levels of MMP-1 and MMP-14, as well as thioredoxin, which suppressed HSC activation [156]. These studies indicated that IC-2 sheets could be promising therapeutic options for treating established hepatic fibrosis.

**Cell-free therapy based on MSCs**

MSCs secrete many molecules into the culture medium (CM), such as cytokines/chemokines, free nucleic acids, EVs, and lipids, effectively repairing tissue injury [157, 158]. These MSC-derived secretomes and EVs avoid the potential tumorigenicity, rejection of cells, emboli formation, undesired differentiation, and infection transmission of MSC-based therapies. MSC-CM inhibits HSC activation by reducing the expression of profibrotic genes such as α-SMA, COL1A1, and MMP2 in vitro, reducing collagen accumulation and inflammation, and increasing hepatocyte survival in a mouse hepatic fibrosis model [159, 160]. In addition, proteomic analysis of MSC-CM revealed a broad spectrum of molecules involved in immunomodulation and liver regeneration in Gal-N-induced liver-injured rats [161].

Notably, EVs are important paracrine factors secreted by MSCs. EVs are membrane-bound vesicles that include apoptotic bodies (50–4000 nm), microvesicles (100–1000 nm), and EXs (40–100 nm) [162]. Accumulating evidence shows that EVs derived from MSCs

**Table 1.** The application of genetically modified MSCs in animal models of liver fibrosis/cirrhosis

| Genetic modification | MSCs sources | Animal models | Dosages | Outcomes | Mechanisms | References |
|---|---|---|---|---|---|---|
| HGF | Bone marrow, umbilical cord | $CCl_4$-induced liver fibrosis in Rats, DMN-induced liver fibrosis in Rats | $2 \times 10^6$ ~ $1 \times 10^7$ | Promote liver function and attenuate liver fibrosis | Upregulate HGF expression; promote hepatocyte proliferation and inhibit hepatocyte apoptosis; decrease collagen; reduce MMP-9, MMP-13, MMP-14 and TIMP-1 expression; decrease fibrogenic cytokines PDGF-bb and TGF-β1 | [247–250] |
| IGF1 | Bone marrow | TAA-induced liver fibrosis in mice | $5 \times 10^5$ | Reduce liver fibrosis | Reduce HSC activation; enhance hepatocyte proliferation; suppress oxidative stress; decrease the expression of pro-inflammatory and pro-fibrogenic genes; activate hepatic macrophages; induce the expression of growth factors and MMP2 in hepatic macrophages | [251], [252] |
| HNF4α | Bone marrow | $CCl_4$-induced liver fibrosis in mice | $1 \times 10^6$ | Improve liver function and inhibit liver cirrhosis | Promote the expression of NF-κB signaling; enhance anti-inflammatory and immune regulatory effects of MSCs | [253] |
| FOXA2 | Bone marrow | $CCl_4$-induced liver fibrosis in rats | / | Improve liver function and inhibit liver fibrosis | Promote hepatic differentiation of MSCs | [254] |
| FGF4 | Bone marrow | $CCl_4$-induced liver fibrosis in rats | / | Improve liver function | Promote the location of MSCs; enhance proliferation of hepatocytes by increasing the expression of PCNA, EpCAM, and Jagged1 | [255] |
| FGF21 | Adipose tissue | TAA-induced liver fibrosis in mice | $1.5 \times 10^6$ | Improve liver function and inhibit liver fibrosis | Reduce serum HA, α-SMA, collagen, and TIMP1; suppress p-JNK, NF-κB, and p-SMAD2/3 signaling pathways | [256] |
| IL10 | Amniotic tissue | TAA-induced liver fibrosis in mice | $1 \times 10^6$ | Improve liver function and ameliorate liver fibrosis | Promote secretion IL10; suppress activation of HSCs and the inflammation in fibrotic liver | [257] |
| SERPINA1 | Adipose tissue | $CCl_4$-induced liver fibrosis in mice | $2.5 \times 10^5$ | Improve the survival time of animals with liver cirrhosis | Promote hepatic differentiation of MSCs | [258] |
| PTP4A1 | Placenta | BDL-induced liver cirrhosis in rats | $2 \times 10^6$ | Alleviate liver fibrosis | Increase ATP production and mitochondrial biogenesis; enhance the metabolism of mitochondria via increased mtDNA and ATP production | [259] |
| SMAD7 | Bone marrow | $CCl_4$-induced liver fibrosis in rats | $3–5 \times 10^6$ | Improve liver function and ameliorate liver fibrosis | Reduce collagen I and III, TGFβ1, TGFBR1, α-SMA, TIMP1, laminin, and hyaluronic acid, increase MMP1. | [260], [261] |
| ECM1 | Hair follicle | $CCl_4$-induced liver fibrosis in mice | $1 \times 10^6$ | Improve liver function | Promote hepatic differentiation of MSCs; inhibit HSC activation; inhibit TGF-β/SMAD signaling pathway | [262] |

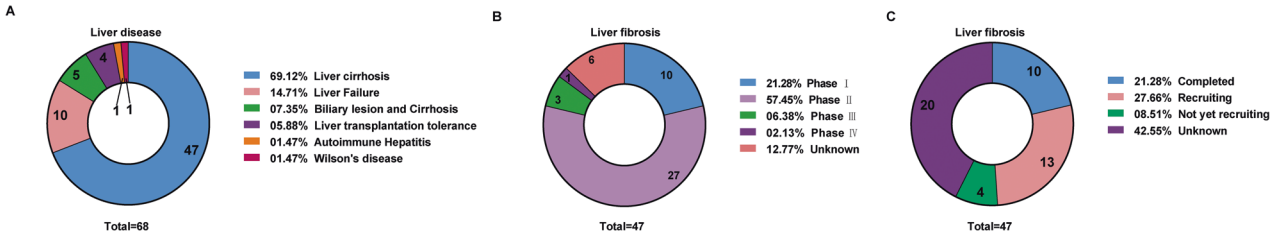


**Fig. 2** Clinical trials of MSCs in liver diseases. **A** An overall of clinical trials based on MSCs classified by liver disease type. **B** Clinical trials of liver cirrhosis based on MSCs classified by the clinical phase. **C** Clinical trials of liver cirrhosis based on MSCs classified by status. The data showed the number and percentage

have therapeutic effects on hepatic fibrosis by reducing inflammation and hepatocyte apoptosis. Human BM-MSC-derived EVs effectively targeted inflammation and reduced fibrosis in a mouse model, which was accompanied by decreased serum levels of ALP, bile acid (BA), and ALT. EVs reduced liver accumulation of granulocytes and T cells and inhibited VCAM-1 expression [163]. Another study showed that EVs from amnion-derived MSCs ameliorated inflammation and fibrogenesis in a rat model of NASH and hepatic fibrosis, potentially by attenuating HSC and KC activation [164]. Similarly, human embryonic stem (ES) cell-derived MSC-EVs exerted immunomodulatory and antiapoptotic effects on TAA-induced chronic rat liver injury by upregulating anti-inflammatory cytokines (TGF-β and IL-10) and antiapoptotic genes (BCL-2) and downregulating major contributors to fibrosis, including COL1A1, α-SMA, and TIMP1 [165].

EXs from MSC-CM have advantages over corresponding MSCs; they are smaller and less complex than cells, so they are easier to produce and store and can potentially avoid some of the regulatory issues associated with MSCs. Several studies have focused on the beneficial therapeutic effects of MSC-derived exosomes in animal models of hepatic fibrosis. An in vivo study showed that hBM-MSC-EXs effectively alleviated hepatic fibrosis, which was characterized by a reduction in collagen accumulation, enhanced liver functionality, inhibition of inflammation, and increased hepatocyte regeneration. hBM-MSC-EX treatment could ameliorate $CCl_4$-induced hepatic fibrosis by inhibiting HSC activation through the Wnt/β-catenin pathway [166]. Tan et al. found that MSC-derived exosomes contained beclin 1 (BECN1), which promoted HSC ferroptosis by suppressing glutathione peroxidase 4 (GPX4) expression. Knockdown of BECN1 in MSCs inhibited ferroptosis and the antifibrotic effects of MSC-derived EXs in HSCs and fibrotic livers [167]. Several studies have indicated that MSC-EXs contain miRNAs such as miR-125b, miR-150-5p, and miR-486-5p, which suppress the activation of HSCs and reduce fibrosis [168–170]. In addition, EXs produced by MSCs could be modified to improve their therapeutic effects on liver failure. MiR-122-modified AD-MSCs expressed high levels of miR-122, which could transfer miR-122 to HSCs and further affect the expression levels of miR-122 target genes, such as insulin-like growth factor receptor 1, cyclin G1, and prolyl-4-hydroxylase α1, which are involved in HSC proliferation and collagen maturation [144]. Tang et al. reported that EXs derived from clinical-grade fibroblast-like MSCs were engineered to carry antisense oligonucleotide targeting STAT3 and could suppress STAT3 levels and ECM deposition in mice with established hepatic fibrosis and significantly improve liver function [171]. These animal model-based studies suggested that EVs may represent effective cell-free therapeutic agents for treating liver diseases. MSC-derived EVs exert beneficial therapeutic effects. However, there are currently no clinical trials on MSC-derived EVs in liver diseases. Many major obstacles, such as the application strategies and therapeutic efficacies, as well as stable and consistent isolation of EVs, remain to be resolved.

## CLINICAL APPLICATIONS OF MSCS IN LIVER CIRRHOSIS TREATMENT

### Overview of MSCs in the treatment of liver cirrhosis in clinical trials

Based on the well-known properties of MSCs, including ease of isolation and expansion in vitro, self-renewal and multiple differentiation capacity, injury tropism, immunomodulation, and low immunogenicity, these cells are considered the most promising candidates for cell therapy. MSCs have been used in clinical trials to treat many diseases [172] related to inflammation and injury. Liver diseases are associated with immune responses and tissue injury. Therefore, liver diseases are ideal targets for MSC therapy. There are 68 clinical trials for diseases involving MSC intervention registered at https://clinicaltrials.gov/ as of October 2022, and most these were focused on liver cirrhosis ($n = 47$) (Fig. 2A). Ten trials had been completed, and only six of them posted results. Thus, limited data are available to evaluate the therapeutic effects of MSCs. We also collected 14 published clinical trial articles on MSC treatment of liver cirrhosis, which overlapped with the 6 completed clinical trials that had results. Therefore, we referred to these 14 published articles to evaluate the outcomes of MSC treatment of liver cirrhosis.

Among the 14 articles, 12 revealed exciting effects of MSCs on liver cirrhosis by evaluating liver function, liver volume, model for end-stage liver disease (MELD) scores, and cytokine expression. Most of these studies followed up with patients for more than 6 months and verified the safety of MSC transplantation. We believe that a Phase II study about HBV-related decompensated liver cirrhosis published in 2021 is the most convincing [173] because it had the longest follow-up and largest sample size. In this study, 219 patients were recruited and divided into a control group ($n = 111$) and a UC-MSC-treated group ($n = 108$). UC-MSCs were transplanted into patients monthly three times in total, and the patients were followed up for 75 months. No obvious side effects were observed, and there was a significantly increased overall survival rate and improved liver function in the UC-MSC-treated group. However, not every study showed positive results. Two clinical trials did not observe any therapeutic effects of MSCs on liver function [174, 175]. These studies recruited 27 patients and 12 patients, respectively. This outcome is probably because the sample size was not very large, and individual differences can affect the results.

Among the clinical trials, two used hepatocytes derived from MSCs to treat liver cirrhosis, which provided another possible MSC application in liver cirrhosis. MSCs were induced to differentiate into hepatocytes by using HGF, DEX, and oncostatin M (OSM) for ~28 days before being injected [176]. After a follow-up of 24 weeks, liver function was improved, as verified by decreases in MELD score, prothrombin complex levels, and serum creatinine and bilirubin and an increase in serum albumin without any adverse effects. However, during the treatment, which was ~2 months, ten patients died because of the severity of their illness. In another phase II trial, the researchers compared the

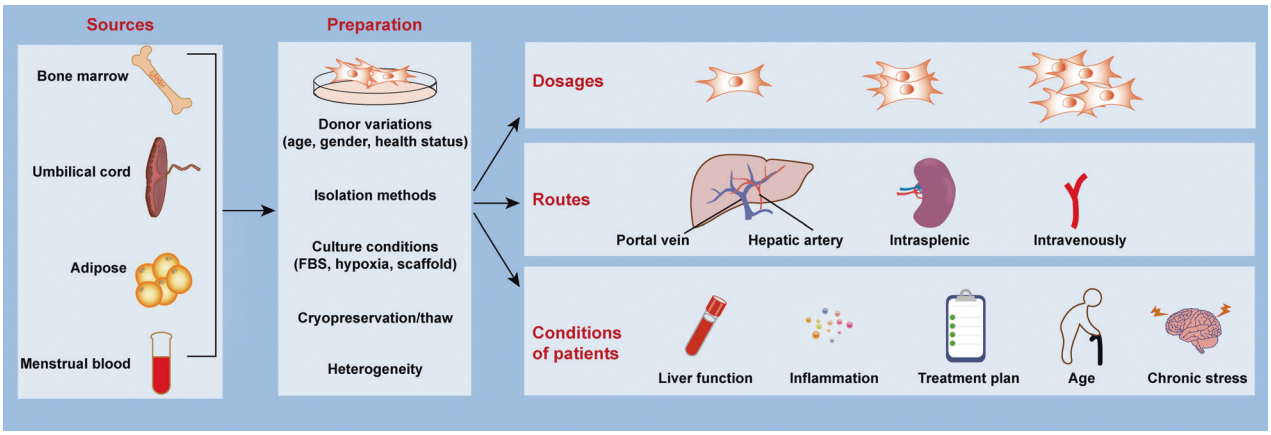


**Fig. 3** The parameters need to be standardized precisely in clinical application of MSCs in liver cirrhosis. Currently, MSCs used in clinical trials for liver cirrhosis treatment are obtained from different sources (bone marrow, umbilical cord, adipose tissue and menstrual blood). During the process of MSC preparation, there are various imprecise parameters, including donor seleciton, isolation methods, culture conditions, cryopreservation and thaw and cell heterogeneity. MSCs are transferred at different dosages and through different routes. All of the above parameters would affect the therapeutic effect of MSCs and disturbing a best standard for clinical application of MSCs is essential for the successful clinical translation of MSCs in the future

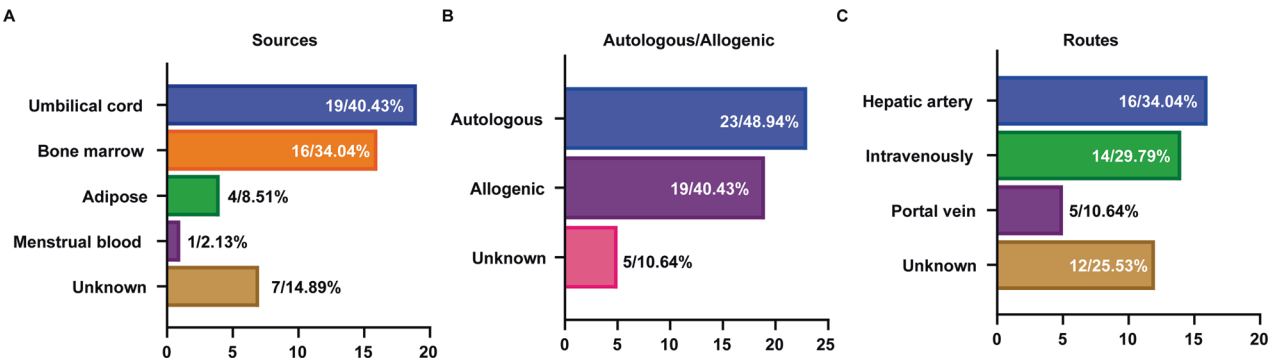


**Fig. 4** Clinical trials of liver cirrhosis based on MSCs application. **A** Clinical trials of liver cirrhosis based on different sources of MSCs. **B** Clinical trials of liver cirrhosis based on autologous and allogeneic transplantation of MSCs. **C** Clinical trials of liver cirrhosis based on transplantation routes of MSCs. The data showed the No. and percentage of corresponding clinical trials

effects of undifferentiated MSCs and hepatocyte-differentiated MSCs [177]. Follow-up of patients at 3 and 6 months post-infusion revealed partial improvements in liver function tests with increases in prothrombin concentrations and serum albumin levels and decreases in the elevated bilirubin levels and MELD scores in both groups. However, statistical comparisons between the undifferentiated and differentiated MSC groups did not indicate any significant differences in clinical and laboratory findings. Animal experiments showed some contradictory conclusions. In a TAA-induced liver cirrhosis model, MSC therapy could regenerate cirrhotic liver tissue and improve liver function and hepatic encephalopathy. Moreover, hepatogenic partially differentiated MSCs were more efficient than undifferentiated MSCs. The superiority of partially differentiated MSCs was most likely due to the increased homing and differentiation abilities of these cells [178]. Hanan El Baz et al. compared the therapeutic effect of undifferentiated human cord blood MSCs, in vitro hepatogenically differentiated MSCs, or freshly isolated rat hepatocytes in a $CCl_4$-induced liver cirrhosis model [179]. The undifferentiated MSC-treated group had better outcomes than the in vitro predifferentiated hepatocyte-like cell-treated group. Considering the outcomes of clinical trials and the animal experiments mentioned above, it is difficult to conclude whether predifferentiation is better. However, we should keep in mind that undifferentiated MSCs have more functions, including immunoregulation and suppressing extracellular matrix accumulation through various mechanisms, and these cells can also secrete HGF, which favors hepatocyte differentiation. To determine a superior strategy for the use of MSCs, more bench research and clinical trials are needed. Furthermore, considering that time is precious to patients, highly efficient MSC differentiation procedures should be optimized if differentiated MSCs are used.

## Parameters that need to be standardized for clinical applications

In these clinical trials of liver cirrhosis, most (57.45%) are in Phase II, and there is only one in Phase IV (Fig. 2B). Only ten clinical trials have been completed (21.28%). Most are recruiting or not yet recruiting (Fig. 2C). The slow progress in the clinical use of MSCs may be due to some imprecise treatment standards. As shown in Fig. 3, there are many clinical parameters used in clinics regarding both cells and patients, including the sources, cell preparation procedure, dosages, engraftment routes of MSCs, and condition of patients, that must be standardized to promote the clinical application of MSCs.

*Sources*. Although MSCs can be isolated from various tissues, such as UC [180], BM [181], AD [182], UC blood [183], placenta [11], amniotic fluid [184], amniotic membrane [185], dental pulp [186], synovium [187], peripheral blood, liver, lung [188], skeletal muscle [189, 190], and hair follicles [191], UC-MSCs and BM-MSCs have been primarily used in clinical trials (Fig. 4A). This is probably

because these cells have been most thoroughly studied and are relatively easy to obtain. Few studies have focused on the therapeutic effects of MSCs derived from different sources in the same study. BM-MSCs are mainly suitable for autologous transplantation. However, there are some limitations to BM-MSC transplantation. Invasive isolation causes injury and inflammation in donors, and the efficiency is low compared with MSCs from other sources [192]. The amount and therapeutic effect of BM-MSCs are also affected by the condition of the donors. For instance, the number and differentiation potential of BM-MSCs decrease with age [193]. Compared with BM-MSCs, UC-MSCs have many advantages. They are easy to obtain and easy to expand to the required amount. UC-MSCs are at an early phase of organic development and show increased self-renewal and differentiation capacity [194]. UC-MSCs show higher hepatic differentiation potential than BM-MSCs, suggesting that UC-MSCs may be advantageous over BM-MSCs for treating end-stage liver disease [195]. In addition, UC-MSCs have lower immunogenicity than bone marrow MSCs [196, 197]. AD-MSCs are also easy to obtain, but these cells have poor proliferation and anti-inflammatory abilities [198]. Therefore, UC-MSCs seem to be the most suitable option for transplantation.

Autologous or allogeneic transplantation is another problem challenging doctors. From the clinical trial data, we can see that there is more autologous transplantation than allogeneic transplantation (Fig. 4B). However, due to the advantages of UC-MSCs, allogeneic transplantation is probably better than autologous transplantation. Donor variations (age, sex, genetics, and health status) are important factors that contribute to the clinical outcomes of MSCs [58]. MSCs isolated from neonatal tissues exhibit a longer active life and better proliferation and differentiation potential than those derived from adult tissues. Furthermore, MSCs derived from unhealthy donors may result in negative clinical outcomes [199].

*Cell preparation*. Several studies have shown that several parameters during cell product preparation affect the clinical outcomes of MSC treatment, including the isolation methods, culture conditions, cryopreservation and thawing, and product heterogeneity [200, 201]. From the beginning, various isolation methods have led to robust differences in the potency of MSCs. For example, techniques used to isolate MSCs from human UC and placenta vary widely, and there are differences in whether the specific sections of perivascular tissues are removed [201]. It was also reported that in UC, MSCs from different parts of the cord were different [202]. Regardless of the isolation method, functional and molecular differences exist among MSCs produced by different centers even when the starting material is the same and MSCs are isolated from aliquots [203]. Therefore, developing a worldwide standard to propagate MSCs is very important.

In general, MSCs should be amplified in vitro for at least several weeks to obtain enough cells for cell therapy; therefore, the culture conditions, including media, serum, growth factors, scaffolds and culture systems, are important to maintain the potency of MSCs [200, 204]. Fetal bovine serum (FBS) is commonly used in research labs to provide nutrients and favor cell adherence. However, there are several severe limitations in the use of FBS to culture clinical-grade MSCs. The risk of contamination and transmission of infectious agents [205], aberrant immune reactions caused by residual animal proteins [206], antibodies against FBS produced by MSCs [207], ethical concerns [208] and batch-to-batch variability [209] make FBS inadequate for culturing MSCs for clinical applications. Several culture media supplements have been developed to replace FBS, including human AB serum, human platelet lysate and chemically defined media (CDM). However, these alternatives have their own limitations [201]. Serum-free, xenobiotic-free CDM is the preferred alternative to FBS and has been shown to improve the differentiation and expansion rates of MSCs [210–212]. The oxygen content of the culture conditions is another important parameter that should be considered. Typically, 20% $O_2$ is used in normal culture conditions in vitro. However, the $O_2$ concentration in the tissue environment of native MSCs ranges between 1% and 7%. High oxygen levels may compromise the therapeutic benefits of MSCs [213]. Culturing MSCs in hypoxic conditions enhances their proliferation, homing, antiapoptotic, and proangiogenic capacities and minimizes spontaneous differentiation [214–216].

Cryogenic banking is needed to store MSCs before transplantation, and alterations in MSCs induced by cryopreservation and subsequent thawing are another challenge regarding the clinical use of MSCs. Cryopreservation has been demonstrated to stunt immunosuppressive capabilities [217], postinfusion biodistribution, engraftment, and clearance kinetics [78, 218]. Furthermore, freeze–thaw steps and transient warming also affect MSC potency [217]. Thawed MSCs exhibit diminished structural integrity upon rewarming, resulting in the cells being recognized and deleted by activated T cells and significantly diminishing the lifetime of MSCs in patients following infusion [78]. However, freshly thawed MSCs can be fully recovered after 1–3 days, at least 24 h of postthaw culture [219, 220].

MSC products have robust heterogeneity not only because of their natural subpopulation diversity but also because of the donor, cell source, and variable parameters during preparation [221–223]. Heterogeneous cells showed different proliferation, differentiation, and immunoregulatory capacities [224, 225]. To overcome the heterogeneity of MSCs and control the therapeutic potency of MSC products, highly homogeneous MSCs were obtained by using induced pluripotent stem cells (iPSCs) [226]. iPSCs have been shown to be safe and effective without any sign of tumorigenesis in a clinical trial of GvHD (NCT02923375). On the other hand, iPSC-MSCs can be used at a low passage number. It has been demonstrated that long-term expansion of human UC-MSCs in vitro (passage 11) reduces their functions [227]. Another study showed that human AD-MSCs at passage 9 exhibited a senescent phenotype and impaired therapeutic effects in a murine hindlimb ischemia model [228]. Expanding hMSCs on soft poly(ethylene glycol) hydrogel matrices [229] and three-dimensional (3D) [230] culture systems is also beneficial for maintaining the early-passage MSC phenotype during expansion. A bioactive hydrogel scaffold has also been proven to improve the therapeutic effect of MSCs [231]. Fibroblast contamination is probably another source of heterogeneity because these cells can express most MSC markers and adhere to plastic [232]. However, the increased expression of CD166 and decreased expression of CD9 in MSCs could be used to purify these cells from fibroblasts by flow-activated cell sorting (FACS) [201]. Taken together, developing best practices for MSC preparation by considering all of the crucial parameters is essential for the successful clinical translation of MSCs.

*Routes of administration*. In clinical trials, various engraftment routes have been used for MSC transplantation, primarily the hepatic artery, followed by intravenous administration and the portal vein (Fig. 4C). There have also been clinical trials transplanting MSCs via the intrasplenic route [233]. It is hard to say which route is the best. A study on acute liver failure concluded that intraportal injection was better for repairing liver injury than hepatic intra-arterial injection, peripheral intravenous infusion, and in situ intrahepatic injection [234]. Unfortunately, no similar study has been performed on liver cirrhosis. We cannot draw a conclusion by comparing different studies.

Considering the convenience and side effects, intravenous injection would be the most convenient and safest route. However, ~60% of cells accumulate in the lung and are cleared by the immune system, as indicated by animal experiments [235]. Thus, more cells should be prepared if the cells will be

transplanted intravenously. Intraportal, intrahepatic, and intrasplenic injection can deliver MSCs faster and avoid off-target effects. However, we should bear in mind the complications of each injection route. These injection routes all require surgery and cause trauma, and some patients will develop a fever [176]. The condition of the patient should also be considered. Therefore, it is difficult to determine which method of administration is optimal. How to choose the injection route should depend on each patient's comprehensive condition.

*Doses*. Dose selection for MSC transplantation in current clinical trials is highly irregular. MSC transplantation is performed according to patient body weight in most clinical trials ($0.5–4 \times 10^6$/kg), while other clinical trials administer MSCs according to the quantity of cells ($1 \times 10^7–5 \times 10^8$ cells). Whether MSC therapy should follow other medicine dosing strategies deserves consideration. Several clinical trials have focused on the effect of different doses, but they have not posted the data yet (NCT02705742, NCT03626090, NCT05080465, NCT05227846, NCT05155657). However, based on the published data, we can see that as few as $1 \times 10^7$ cells administered by intrasplenic injection have apparent therapeutic effects on liver cirrhosis for six months [233].

Most clinical trials transplant MSCs in one dose, while others transplant MSCs in several doses, ranging from two to four doses with different intervals. One Phase II study compared the effect of single and two-time transplantation with a monthly interval. The results showed no significant difference between single and double transplantation [236]. Another study showed that one dose of MSCs could improve liver function for up to two years of follow-up. No significant therapeutic effect was observed after 2 years [237]. It was reported that multiple infusions of MSCs may enhance the effect of transplantation in animal experiments [238, 239]. Based on these studies, several injections with long intervals are probably beneficial for improving the therapeutic effect of MSCs.

*Patient condition*. We noticed no trials grouping patients according to their condition in the clinical trials and published clinical articles. The properties of MSCs could be affected by their microenvironment to a great extent, including migration, differentiation, and immunoregulation. MSCs and the microenvironment, especially the inflammatory microenvironment, have substantial crosstalk and interactions [58]. Therefore, the microenvironment of patients is a crucial parameter that needs to be considered in clinical trials. The cause of liver cirrhosis, the inflammation level, liver function and the treatment strategies affect the microenvironment of the patient [240, 241]. It should be mentioned that patient age is another important parameter that has been demonstrated to affect the therapeutic effect of MSCs [242]. Based on animal experimental data, we can also see that the age of the host adversely affects hMDSC-mediated bone regeneration [243]. In most clinical trials, the enrolled patients are ~18–30 years old to 65–80 years old (clinicaltrials.gov). This age span is too broad, resulting in significant differences in the microenvironment within a single group. The aging microenvironment, including the senescence-associated secretory phenotype (SASP), has been proven to impair the function of transplanted MSCs [244, 245]. We can obtain much more accurate results if patients are grouped by narrow age ranges. In one previous study, we found that chronic stress inhibited the therapeutic effects of MSCs in a mouse model [246]. Another study showed that DEX abrogated the therapeutic effects of MSCs on fibrin deposition, serum levels of bilirubin, albumin, and aminotransferases, and T-lymphocyte infiltration [123]. Combining the comprehensive conditions of patients and the therapeutic effects, we can evaluate whether a patient is suitable for MSC treatment before MSC transplantation. We can also do something to keep the patients in the best condition to receive MSC transplantation.

## CONCLUSION

In this review, we examined the paradoxical roles of MSCs in the pathogenesis and treatment of hepatic fibrosis/cirrhosis. On the one hand, MSCs are involved in the pathogenesis of hepatic fibrosis through various mechanisms, such as differentiation into MFs and the promotion of HSC activation. MSCs can also affect hepatic fibrosis by regulating the inflammatory microenvironment. However, in animal experiments and clinical trials, MSCs are used to treat hepatic fibrosis/cirrhosis. Increasing evidence has shown that MSC transplantation can alleviate hepatic fibrosis and improve liver function through several mechanisms, including hepatocyte differentiation and protection, inhibiting HSC activation, antifibrotic factors, and immunoregulation. The contradictory effects of MSCs on hepatic fibrosis mainly result from whether these cells are endogenous or exogenous. Most of the data suggesting the involvement of MSCs in the pathogenesis of hepatic fibrosis were obtained from endogenous MSCs. However, the antifibrotic effects of MSCs were mainly observed after the transplantation of exogenous MSCs. Thus, the long-term fibrotic microenvironment alters the properties and functions of MSCs.

Based on studies on MSCs in hepatic fibrosis regression, several new methods to enhance the therapeutic effects of MSCs have been developed, including pretreatment, gene modification, cell-free therapy, cell sheets, and spheroids. Further study is warranted to determine whether these are new strategies for use in the clinic. However, it is necessary to perform more bench research to verify the validity and safety of these new strategies.

MSCs are the most promising candidates for cell therapy due to their many advantages. Although some satisfactory outcomes in some patients with cirrhosis have been observed in clinical trials, various parameters need to be optimized from cells to recipients, including the sources and preparation of MSCs and the routes and doses for MSC transplantation. Patient condition, which is much easier to ignore, is another important factor that affects the therapeutic efficacy of MSCs. However, more future work needs to be done to advance the development of MSC therapy.

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## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

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